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13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*
53BP1 has been reported to interact with the central DNA-binding domain of the tumor suppressor p53 and to enhance p53-dependent transcription. The C-terminus of 53BP1 contains a tandem BRCT motif. This domain was first identified in the C-terminal region of BRCA1 and has since been found in several proteins involved in various aspects of cell cycle control, recombination and DNA repair. The interaction with p53 and sequence homology with BRCA1 raised the possibility that 53BP1 may be involved in the maintenance of genomic stability. Western blot and immunofluorescence studies showed that upon DNA damage 53BP1 becomes hyperphosphorylated and forms discrete nuclear foci at the sites of DNA lesions. Co-immunostaining analyses revealed that these foci co-localize with BRCA1 foci several hours after DNA damage. Furthermore, small amounts of 53BP1 can be co-immunoprecipitated with BRCA1 after irradiation but not in untreated cells. Like BRCA1, 53BP1 becomes phosphorylated by ATM *in vivo* following ionizing radiation. Both proteins were also found to localize to stalled replication forks in response to replicational stress. These findings indicate that 53BP1 is involved in the early response to genotoxic stress. Given its interactions with p53 and BRCA1 it is reasonable to predict that 53BP1 might also act as a tumor suppressor.

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INTRODUCTION

53BP1 (tumor suppressor p53-binding protein 1) has been identified in a yeast two hybrid screen as protein that interacts with the central DNA-binding domain of p53. Similar to BRCA1(1),(2),(3). 53BP1 has been reported to enhances p53-mediated transcriptional activation(4). The interaction with p53 is mediated by the C-terminus of 53BP1 which contains two BRCT motifs(5),(6),(7). These motifs were first identified in the C-terminal region of BRCA1 and have since been found in several proteins involved in various aspects of cell cycle control, recombination and DNA repair. The interaction with p53 and sequence homology with BRCA1 prompted us to investigate whether 53BP1 participates in the control and maintenance of genomic stability.
Several significant findings which contribute to the knowledge in the DNA damage response field, are summarized below.

BODY

To get insight into the function of 53BP1, I first compared the localization of 53BP1 before and after DNA damage with that of BRCA1 (*Statement of Work, Task 1*). BRCA1, the product of the breast cancer susceptibility gene 1, is known to be cell cycle regulated and to form nuclear foci in S-phase cells. S-phase foci disperse in response to 10-50 Gy of irradiation and new foci appear several hours later in the majority of cells. These so-called ionizing radiation-induced foci (IRIF) co-localize with the NBS1/MRE11/Rad50 complex and Rad51(8),(9),(10),(11),(12),(13). Using fluorescent microscopy I did not observe 53BP1 foci in S-phase cells nor did the 53BP1 protein level change during the cell cycle as assessed by Western Blotting. However, 53BP1 rapidly formed distinct nuclear foci in response to ionizing radiation and these foci co-localized with BRCA1 and Rad51 several hours after radiation (Fig.1).

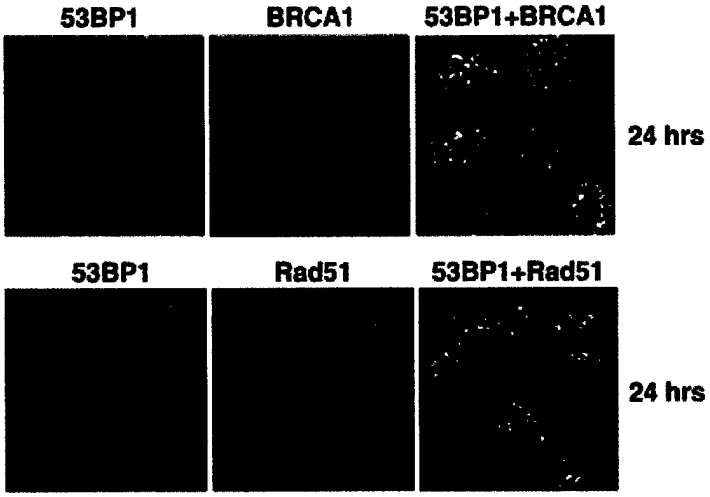

Figure 1: 53BP1 co-localizes with BRCA1 and Rad51 following γ-irradiation. HeLa cells were co-immunostained with anti-53BP1 and anti-BRCA1 or anti-Rad51 24 hours after exposure to 10 Gy of γ-irradiation.

Moreover, co-immunoprecipitation experiments revealed small amounts of 53BP1 co-immunoprecipitated with BRCA1 in cell extracts prepared several hours after exposure to 50 Gy of irradiation. No 53BP1 was detected in BRCA1 immunoprecipitates of untreated cells (Fig.2) suggesting that 53BP1 and BRCA1 only interact upon DNA damage.

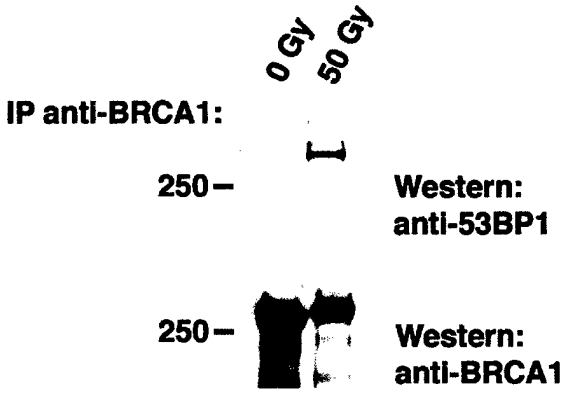

Figure 2: Co-immunoprecipitation of 53BP1 and BRCA1 following DNA damage. K562 cells were exposed to 0 or 50 Gy of γ-radiation 30 hours prior to immunoprecipitation with affinity-purified anti-BRCA1 polyclonal antibodies. The samples were separated on 4-15% SDS-PAGE and immunoblotted with anti-53BP1 antibodies prior to re-blotting with anti-BRCA1 antibodies as indicated.

This differs from the interaction of 53BP1 and p53. P53 can be co- immunoprecipitated with 53BP1 (and vice versa) in untreated cells but does not re-localize with 53BP1 to irradiation-induced foci.

Co-localization studies using phospho-specific anti-histone H2AX antibodies could pin point the irradiation-induced 53BP1 foci to the sites of DNA strand breaks (see appended publication (14)). The number of these foci peaked at about 20-40 minutes after irradiation and then gradually decreased coinciding with the reported time course of DNA repair. Moreover, the number of 53BP1 foci increased with the dose of γ-radiation and correlated with the calculated number of irradiation-induced double strand breaks. These findings strongly suggested that 53BP1 is involved in DNA damage recognition and/or repair pathways (see appended publication (14)). Given the rapid re-localization of 53BP1 to DNA lesions followed by the slower co-localization and interaction with BRCA1, I was wondering whether 53BP1 is involved in the recruitment of BRCA1 to DNA strand breaks.

To address this question I designed various 53BP1 deletion constructs to map the regions required for 53BP1 foci formation and BRCA1 interaction (*Statement of Work, Task 2*). The proposed EGFP and RFP tags proved to be inapt to monitor real time localization. The EGFP fluorescence was too weak and the RFP tags formed oligomers. However, using cells transiently transfected with HA tagged 53BP1 constructs I could confine a 580 aa region upstream of the C-terminal BRCT repeats which is required and sufficient for 53BP1 foci formation (Fig.3).

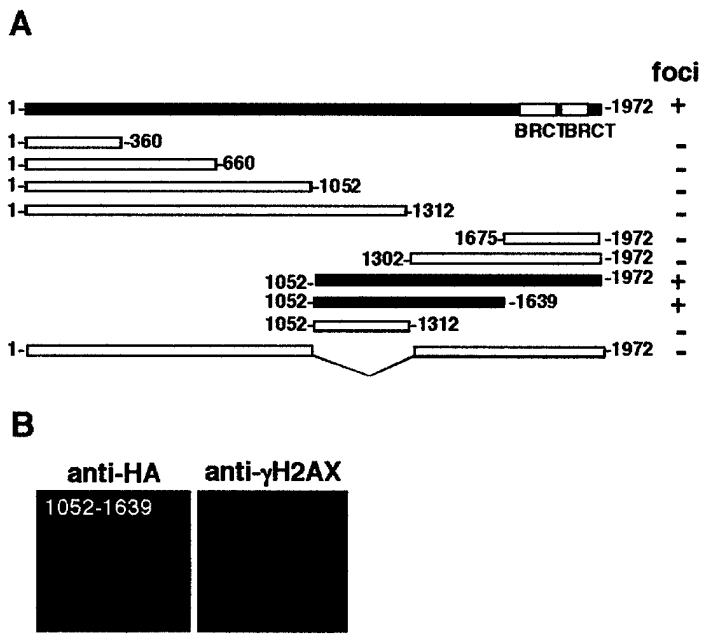

Figure 3: A C-terminal region of 53BP1 is necessary and sufficient for the damage-induced focus-localization of 53BP1. (A) Schematic diagram of wild-type 53BP1 and its derivatives. The BRCT motifs are indicated, Wild-type or mutants 53BP1 were N-terminally fused to a HA-tag and NLS and transiently expressed in U2OS cells. Foci formation in response to γ-radiation was tested by IF using anti-HA antibody. (B) Cells were irradiated with 1 Gy, fixed and co-immunostained with anti-HA antibody and control anti-γ-H2AX antibody.

I am currently generating stable transfectants to define the region critical for the interaction with BRCA1 and to test whether this interaction depends on 53BP1-P. 53BP1 becomes hyperphosphorylated in response to DNA damage and this phosphorylation is ATM dependent (see appended publication (14)). Using a peptide screening method I had identified four sites at the 53BP1 N-terminus (S6, S25, S29, S784) that are phosphorylated by ATM *in vitro* (Fig. 4).

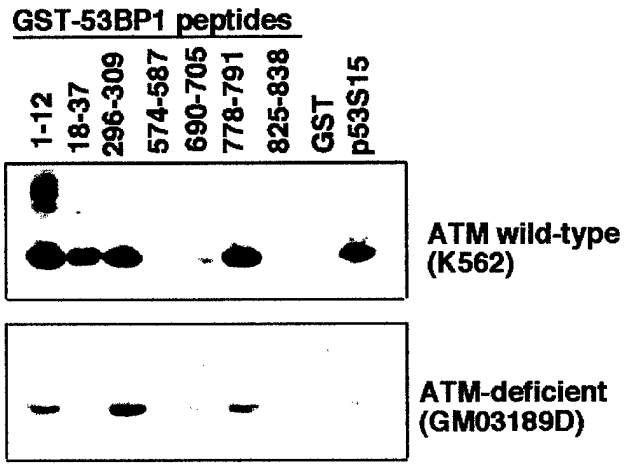

Figure 4: In vitro mapping of ATM phosphorylation sites. ATM kinase assays were performed using 53BP1 GST fusion peptides as substrates and ATM kinase immuno-precipitated from K562 lysates or from negative control GM03189d lysates. GST or a GST fusion peptide containing 14 amino acids surrounding the Ser-15 of p53 were used as negative and positive controls, respectively.

Phospho-specific antibodies raised against two of these sites (S25/S29) confirmed that 53BP1 is phosphorylated by ATM *in vivo* following DNA damage (Fig. 5). Using phospho-mutants I will assess whether 53BP1 phosphorylation is required for recruitment of BRCA1 to foci.

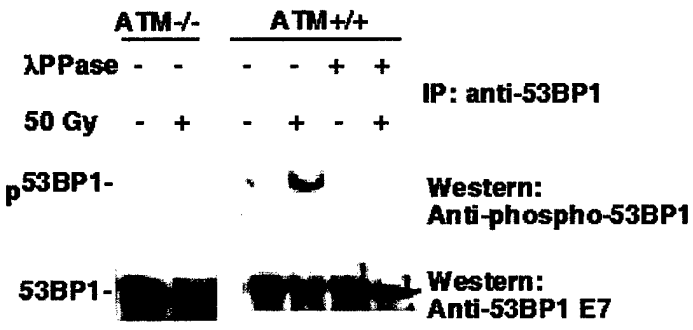

Figure 5: 53BP1 is phosphorylated *in vivo* following ionizing radiation. 293T cells containing wild-type ATM (ATM+/+) and ATM-deficient GM03189D cells (ATM-/-) were mock treated or irradiated (50 Gy). After 1 hour, whole cell lysates were immuno-precipitated with anti-53BP1 antibody E7. Duplicate samples were treated with lambda phosphatase. Western blots were performed with anti-53BP1 E7 or anti-phospho-53BP1 antibodies (raised against the phospho-serines 25/29 of 53BP1).

So far, no animal model was available to study the function of 53BP1 in tumorigenesis. To facilitate our understanding of 53BP1 and 53BP1/BRCA1 interaction, I designed a 53BP1 knock out construct and initiated the generation of 53BP1-deficient mice. These mice as well as cell lines derived from them are now available in our lab and will allow me to study the interactions between 53BP1, BRCA1 and especially p53 in more detail. Preliminary results are very exciting and suggest that 53BP1 is a powerful tumor suppressor.

KEY RESEARCH ACCOMPLISHMENTS

- 53BP1 is involved in DNA damage signaling pathways.
- 53BP1 co-localizes and interacts with BRCA1 in response to DNA damage.
- 53BP1 and BRCA1 form foci at the sites of DNA strand breaks.
- A region upstream of the BRCT repeats is required for 53BP1 foci formation.
- 53BP1 is phosphorylated by ATM at S25/S29 in response to ionizing radiation.

REPORTABLE OUTCOMES

I. Rappold, K. Iwabuchi, T. Date, J. Chen, *J Cell Biol* **153**, 613-20. (2001).

I. M. Ward, X. Wu, J. Chen, *J Biol Chem* **276**, 47755-8. (2001).

I. M. Ward, J. Chen, *J Biol Chem* **276**, 47759-62. (2001

CONCLUSIONS

In summary, the findings demonstrate that 53BP1 plays an important role in the surveillance and/or repair of DNA damage. Defects in this sophisticated system are involved in the pathogenesis of many types of cancer. Unraveling 53BP1 function and the underlying molecular mechanism will contribute to our understanding of tumorigenesis.

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Report

Tumor Suppressor p53 Binding Protein 1 (53BP1) Is Involved in DNA Damage–signaling Pathways

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Abstract. The tumor suppressor p53 binding protein 1 (53BP1) binds to the DNA-binding domain of p53 and enhances p53-mediated transcriptional activation. 53BP1 contains two breast cancer susceptibility gene 1 COOH terminus (BRCT) motifs, which are present in several proteins involved in DNA repair and/or DNA damage–signaling pathways. Thus, we investigated the potential role of 53BP1 in DNA damage–signaling pathways. Here, we report that 53BP1 becomes hyperphosphorylated and forms discrete nuclear foci in response to DNA damage. These foci colocalize at all time points with phosphorylated H2AX (γ-H2AX), which has been previously demonstrated to localize at sites of DNA strand breaks. 53BP1 foci formation is not restricted to γ-radiation but is also detected in response to UV radiation as well as hydroxyurea, camptothecin, etoposide, and methylmethanesulfonate treatment. Several observations suggest that 53BP1 is regulated by ataxia telangiectasia mutated (ATM) after DNA damage. First, ATM-deficient cells show no 53BP1 hyperphosphorylation and reduced 53BP1 foci formation in response to γ-radiation compared with cells expressing wild-type ATM. Second, wortmannin treatment strongly inhibits γ-radiation–induced hyperphosphorylation and foci formation of 53BP1. Third, 53BP1 is readily phosphorylated by ATM in vitro. Taken together, these results suggest that 53BP1 is an ATM substrate that is involved early in the DNA damage–signaling pathways in mammalian cells.

Key words: 53BP1 • DNA damage • nuclear foci • γ-H2AX • ATM

Introduction

Cells have evolved various sophisticated pathways to sense and overcome DNA damage as a mechanism to preserve the integrity of the genome. Environmental attacks like radiation or toxins, as well as spontaneous DNA lesions, trigger checkpoint activation and consequent cell cycle arrest and/or apoptosis. One key protein that coordinates DNA repair with cell cycle progression and apoptosis is the tumor suppressor protein p53. P53 is activated and posttranslationally modified in response to DNA damage (Appella and Anderson, 2000). These modifications include phosphorylation by ataxia telangiectasia mutated (ATM),[1] a protein kinase implicated in DNA damage–signaling pathways (Canman et al., 1998; Khanna et al., 1998). By transcriptionally activating genes involved in cell cycle control, DNA repair, and apoptosis, p53 participates in the maintenance of the genomic integrity after DNA damage.

P53 interacts with p53 binding protein 1 (53BP1). 53BP1 has been identified in a yeast two hybrid screen as a protein that interacts with the central DNA–binding domain of p53 (Iwabuchi et al., 1994). Similar to breast cancer susceptibility gene 1 (BRCA1; Ouchi et al., 1998; Zhang et al., 1998a; Chai et al., 1999), 53BP1 enhances p53-dependent transcription (Iwabuchi et al., 1998). Interestingly, the COOH terminus of 53BP1 contains tandem BRCA1 COOH terminus (BRCT) motifs. This motif was first identified in the COOH-terminal region of BRCA1 and has since been found in a large number of proteins involved in various aspects of cell cycle control, recombination, and DNA repair in mammals and yeast (Koonin et al., 1996; Bork et al., 1997; Callebaut and Mornon, 1997). The func-

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[1]*Abbreviations used in this paper:* ATM, ataxia telangiectasia mutated; BRCA1, breast cancer susceptibility gene 1; BRCT, BRCA1 COOH terminus; DNA-PK, DNA-dependent protein kinase; 53BP1, binding protein 1; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol 3–kinase.

tion of the BRCT domain is not known. However, evidence suggests that BRCT domains may mediate protein–protein interactions (Zhang et al., 1998b).

The presence of BRCT domains in 53BP1 and the reported interaction with p53 prompted us to investigate whether 53BP1 is involved in DNA damage–response pathways. Here we report that 53BP1 becomes hyperphosphorylated and rapidly relocates to the sites of DNA strand breaks in response to ionizing radiation. 53BP1 foci formation is reduced in ATM-deficient cells and can be inhibited by wortmannin in ATM wild-type cells. Moreover, radiation-induced hyperphosphorylation of 53BP1 is absent in cells treated with wortmannin, as well as in ATM-deficient cells. Taken together, these results strongly suggest that 53BP1 participates in DNA damage–signaling pathways and is regulated by ATM after γ-radiation.

Materials and Methods

Cell Culture and Treatments with DNA-damaging Agents

Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. FT169A and YZ5 cells were provided by Dr. Y. Shilon (Tel Aviv University, Ramat Aviv, Israel). Cells grown on coverslips were irradiated in a JL Shepherd ^{137}Cs radiation source at a rate of 1 Gy/min for doses of 1–5 Gy or 10 Gy/min for a dose of 10 Gy. UV light was delivered in a single pulse (50 J/m^2) using a Stratalinker UV source (Stratagene). Before UV irradiation, the culture medium was removed and the medium was replaced immediately after irradiation. All cells were returned to the incubator for recovery and harvested at the indicated times. Genotoxic agents and other drugs were used at the indicated concentrations. After a 1-h exposure, the cells were harvested for immunostaining.

Immunoprecipitation, Immunoblotting, and Immunostaining

Immunoprecipitation, immunoblotting, and immunostaining were performed as described previously (Scully et al., 1997). Rabbit polyclonal anti-53BP1 serum E7 was raised against a glutathione *S*-transferase (GST) fusion protein encoding residues 338–671 of 53BP1. Mouse anti-53BP1 monoclonal antibodies were raised against a mix of three GST fusion proteins encoding residues 1–337, 338–671, and 1,331–1,664, respectively, of 53BP1. Antiphospho-H2AX antibody was generated as described previously (Rogakou et al., 1999).

ATM Kinase Assay

ATM was immunoprecipitated from K562 cells using anti-ATM antibody Ab3 (Oncogene Research Products). Aliquots of the ATM–protein A Sepharose immunocomplexes were resuspended in 25 μl kinase buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 nM ATP) and incubated for 20 min at 30°C with 10 μCi of [γ^{32}]P-ATP and 1 μg of various affinity-purified GST fusion proteins containing different fragments of 53BP1.

Results

53BP1 Forms Nuclear Foci in Response to Various Types of DNA Damage

Several proteins, including BRCA1 and Mre11/Rad50/Nbs1, form DNA damage–regulated, subnuclear foci in the cell. To determine whether 53BP1 participates in DNA damage–signaling pathways, we examined 53BP1 localization after various types of DNA damage using several anti-53BP1 polyclonal and monoclonal antibodies generated for this study. All antibodies specifically recognize endogenous, as well as HA-tagged, full-length 53BP1 as examined by Western blotting, immunoprecipitation, and immunostaining (data not shown). As shown in Fig. 1, 53BP1 is diffusely localized in the nuclei of normal cells, but relocates to discrete subnuclear foci structures in response to ionizing radiation (e.g., 1 Gy). These 53BP1 foci can be detected as early as 5 min after irradiation (data not shown). Higher doses of radiation (e.g., 10 Gy) lead to more but smaller 53BP1 foci (Fig. 1). The number of foci reaches a peak at ~30 min after radiation. Thereafter, the foci number slowly decreases, whereas the foci size increases (data not shown).

Foci formation is also observed in response to other DNA-damaging events. UV radiation induced the formation of numerous small foci, similar to that induced by 4NQO (a UV-mimetic agent) and hydroxyurea (Fig. 1). Treatment with the DNA topoisomerase I poison camptothecin or the topoisomerase II poison etoposide (VP16), which cause DNA single strand and double strand breaks, respectively, also resulted in the formation of 53BP1 foci. Similar results were obtained with the alkylating agent methylmethanesulfonate. However, cisplatin, a DNA cross-linking agent, induced only a few 53BP1 foci during the first hour after drug application, whereas the protein kinase inhibitor UCN-01 and the antimitotic agent paclitaxel (Taxol; Bristol-Meyers Squibb Co.) did not induce 53BP1 foci formation. Thus, different types of DNA damage trigger the recruitment of 53BP1 into discrete nuclear foci.

53BP1 Colocalizes with γ-H2AX in Response to DNA Damage

The time course of 53BP1 foci formation and disappearance is very similar to that recently described for phosphorylated H2AX (Rogakou et al., 1999; Paull et al., 2000). H2AX is one of the histone H2A molecules in mammalian cells and becomes rapidly phosphorylated after exposure of cells to ionizing radiation (Rogakou et al., 1999; Paull et al., 2000). Phosphorylated H2AX (γ-H2AX) appears within 1–3 min as discrete nuclear foci on sites of DNA double strand breaks (Rogakou et al., 1999). Similar to γ-H2AX (Rogakou et al., 1999), the number of 53BP1 foci showed a linear relationship with the severity of DNA damage (Fig. 1 and data not shown). As shown in Fig. 2 A, damage-induced 53BP1 foci colocalized with γ-H2AX at the various time points analyzed. The number of 53BP1 foci was identical to that of γ-H2AX throughout the course of the experiment. In addition, coimmunoprecipitation analysis revealed that 53BP1 and γ-H2AX biochemically interact after γ-radiation (Fig. 2 B). Small amounts of 53BP1 were detected in γ-H2AX immunoprecipitates prepared from irradiated HBL100 cells. In unirradiated cells, H2AX was not phosphorylated and anti–γ-H2AX antibodies did not immunoprecipitate any phosphorylated H2AX. Similarly, 53BP1 was also not present in anti–γ-H2AX immunoprecipitates prepared from unirradiated cells. These results demonstrate that 53BP1 colocalizes and interacts with γ-H2AX at the sites of DNA strand breaks after γ-radiation.

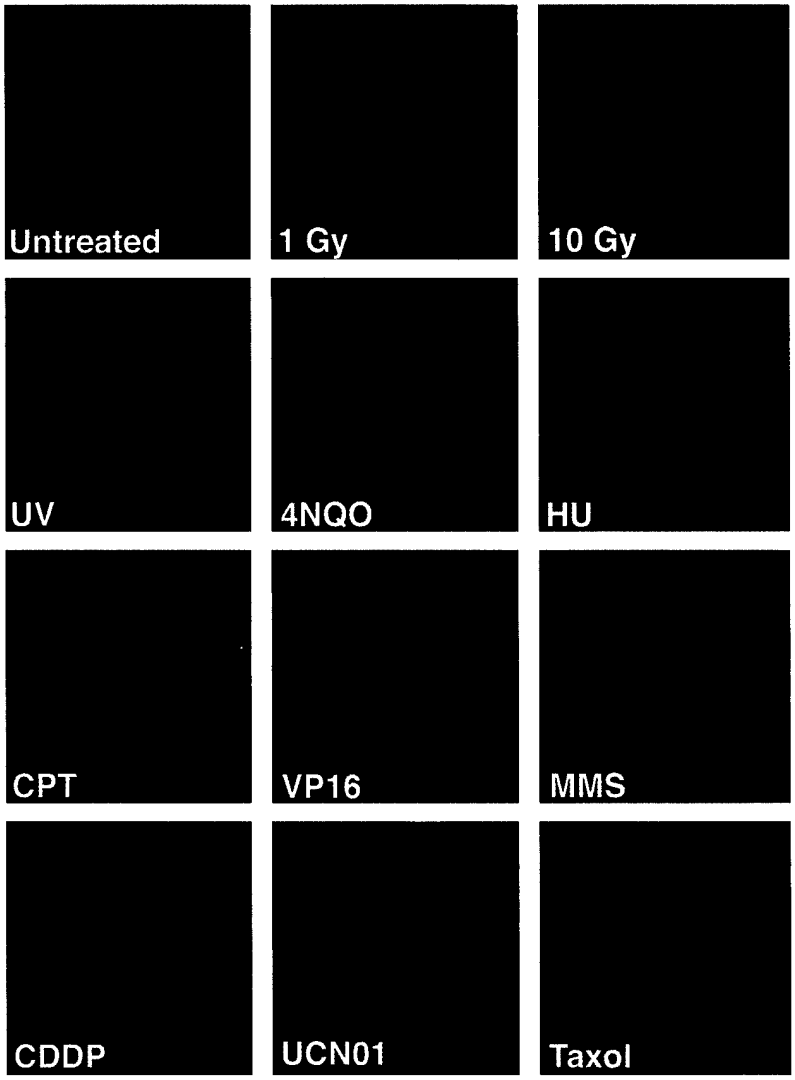

Figure 1. 53BP1 forms nuclear foci in response to DNA damage. HeLa cells were exposed to γ-irradiation (1 and 10 Gy) or a 50 J/m^2 UV pulse 1 h before immunostaining with anti-53BP1 mAb BP13. Alternatively, cells were treated for 1 h with the following drugs: 2 μg/ml 4-nitroquinoline 1-oxide (4NQO), 1 mM hydroxyurea (HU), 1 μM camptothecin (CPT), 40 μg/ml etoposide (VP16), 0.01% methylmethanesulfonate (MMS), 40 μM cisplatin (CDDP), 1 μM 7-hydroxystaurosporine (UCN-01), and 1 μM paclitaxel (Taxol).

ATM Is Involved in 53BP1 Foci Formation

Several phosphatidylinositol 3–kinase (PI3K)-related kinases, including DNA-dependent protein kinase (DNA-PK), ATM, and ATM-related kinase (ATR), participate in DNA damage–responsive pathways (Smith and Jackson, 1999; Khanna, 2000). It is possible that DNA damage–induced 53BP1 foci formation may depend on one or more of these PI3K-like kinase family members.

We first examined 53BP1 foci formation in the presence or absence of DNA-PK using two derivatives of the human glioma cell line MO59 (Lees-Miller et al., 1995). No difference in the time course of 53BP1 foci appearance and disappearance was observed in these two cell lines after exposure to 1 Gy of γ-radiation (data not shown). However, comparison was hampered by the high number of 53BP1 foci in unirradiated MO59K and MO59J cells and subtle differences might be overlooked.

We then examined whether the 53BP1 response to ionizing radiation is affected in cells lacking ATM. Immortalized ATM-deficient fibroblasts (FT169A) were compared with their isogenic derivative cells, YZ5, that have been reconstituted with wild-type ATM cDNA (Ziv et al., 1997). As shown in Fig. 3 A, although irradiation with 1 Gy resulted in a rapid formation of 53BP1 foci in the ATM-reconstituted cells (ATM+), a reduced response was observed in the cells lacking wild-type ATM (ATM−). Similar results were obtained when we compared other ATM-deficient fibroblast lines (GM03189D and GM05849C) with wild-type ATM cell lines (GM02184D and GM00637H) (Fig. 3 A). The time course of the number of 53BP1 foci per cell, as calculated from three independent experiments using YZ5 versus parental FT169A cells, is illustrated in Fig. 3 B.

To further corroborate the role of ATM in 53BP1 foci formation, we pretreated HeLa cells for 30 min with wortmannin before exposure to 1 Gy of irradiation. Wortmannin is a potent inhibitor of the PI3K-related kinases, including ATM and DNA-PK (Sarkaria et al., 1998). As shown in Fig. 3 C, pretreatment with 50 μM wortmannin greatly reduced the number of 53BP1 foci evident 1 h after γ-radiation. At an even higher dose (200 μM), wortmannin completely blocked 53BP1 foci formation. These results suggest that the kinase activities of ATM or other PI3K-related kinases are required for 53BP1 foci formation.

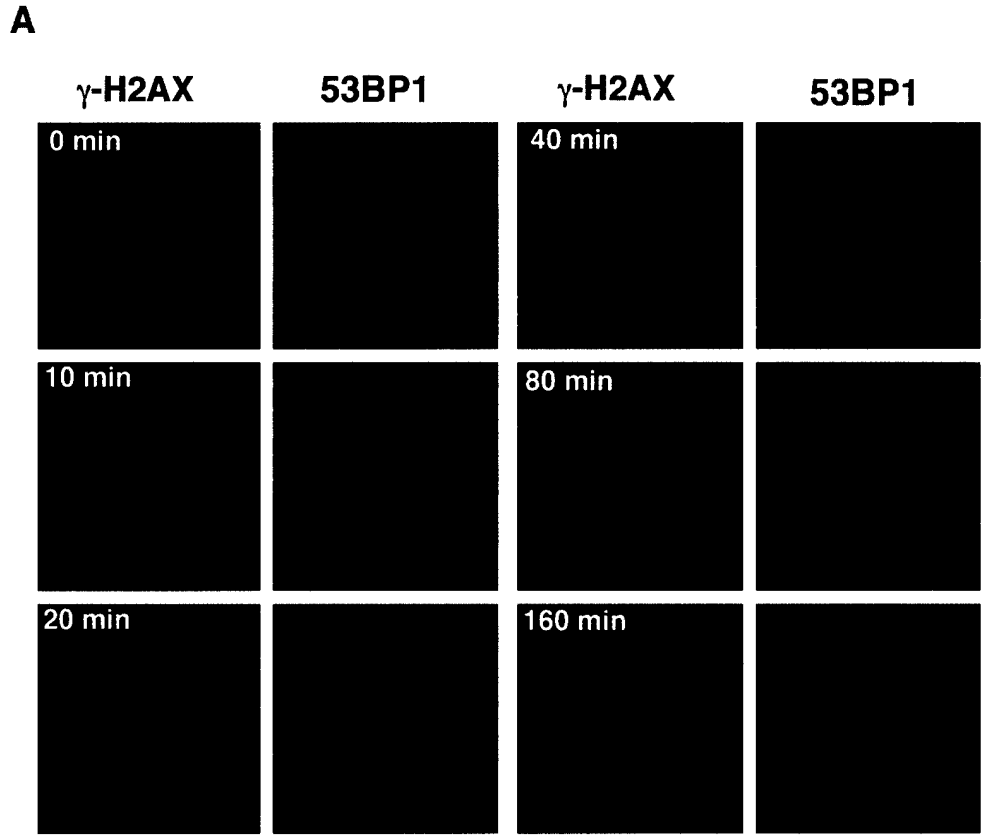

Figure 2. (A) 53BP1 colocalizes with γ-H2AX in response to γ-radiation. WI38 cells were coimmunostained with anti-53BP1 antibody BP13 and affinity-purified anti–γ-H2AX serum before (0 min) and at the indicated time points after exposure to 1 Gy (10–160 min). (B) Coimmunoprecipitation of 53BP1 and γ-H2AX after DNA damage. HBL100 cells were exposed to 0 or 20 of Gy γ-radiation 1 h before lysis in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8, 0.5% NP-40) including 0.3 M NaCl. Immunoprecipitation experiments were performed using anti-γH2AX or anti-53BP1 antibodies. A fivefold higher amount of cell lysate was used for anti–γ-H2AX immunoprecipitation than that used for anti-53BP1 immunoprecipitation. The samples were separated on 4–15% SDS-PAGE and Western blotting was performed using either anti–γ-H2AX or anti-53BP1 antibodies as indicated.

ATM Is Required for DNA Damage-induced Hyperphosphorylation of 53BP1

Many proteins involved in DNA damage–response and/or DNA repair are phosphorylated upon DNA damage. To examine whether 53BP1 becomes phosphorylated in response to γ-radiation, K562 cells were irradiated (20 Gy) and harvested 1 h later. After immunoprecipitation using anti-53BP1 antisera, the samples were incubated for 1 h at 30°C in the presence or absence of λ protein phosphatase and separated on a 3–8% gradient SDS gel. Phosphatase treatment of unirradiated K562 cells revealed a faster migrating form of 53BP1 (Fig. 4 A). This indicates that 53BP1 is modified by phosphorylation in normal undamaged cells. Upon γ-radiation, 53BP1 showed an even slower mobility that was reversed by phosphatase treatment (Fig. 4 A). These results suggest that 53BP1 is phosphorylated in undamaged cells and becomes hyperphosphorylated after γ-radiation.

Since 53BP1 is hyperphosphorylated after γ-radiation, we then examined whether wortmannin would affect radiation-induced 53BP1 phosphorylation. As illustrated in Fig. 4 B, there was no detectable radiation-induced 53BP1 mobility shift in wortmannin (50 μM)-pretreated cells. In contrast, the radiation-induced 53BP1 mobility shift was

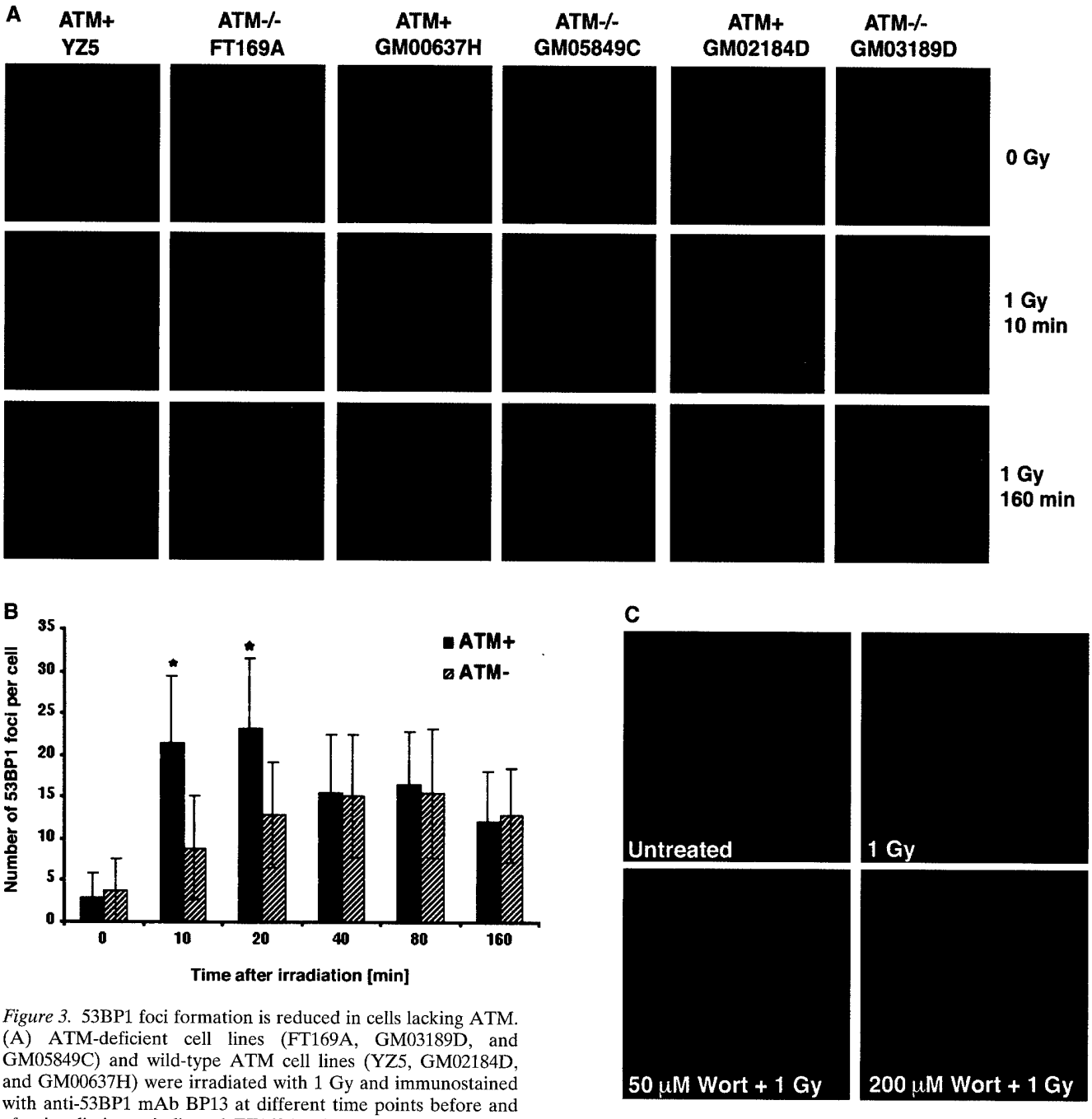

Figure 3. 53BP1 foci formation is reduced in cells lacking ATM. (A) ATM-deficient cell lines (FT169A, GM03189D, and GM05849C) and wild-type ATM cell lines (YZ5, GM02184D, and GM00637H) were irradiated with 1 Gy and immunostained with anti-53BP1 mAb BP13 at different time points before and after irradiation as indicated. FT169A cells and YZ5 cells are isogenic, whereas the other lines are unrelated. (B) The numbers of 53BP1 foci in at least 75 FT169A (ATM−) and YZ5 (ATM+) cells were counted for each time point. Data represent mean ± SD of three independent experiments. The difference in foci number between ATM+ and ATM− cells 10 or 20 min after irradiation was significant at $P < 0.001$ using a Student's t test. (C) Wortmannin inhibits γ-radiation–induced 53BP1 foci formation. HeLa cells were pretreated for 30 min with 0, 50, or 200 μM wortmannin before exposure to 1 Gy of γ-radiation. After recovery for 1 h, the control or irradiated cells were immunostained with anti-53BP1 antibodies.

readily detected in cells that had received no drug treatment before radiation. We next repeated the experiment using the ATM-deficient GM03189D and GM02184D cells expressing wild-type ATM. Again, in ATM wild-type cells, γ-radiation induced a 53BP1 mobility shift in control, but not in wortmannin-pretreated samples (Fig. 4 C). However, no radiation-induced 53BP1 mobility shift was observed in ATM-deficient cells, with or without wortmannin treatment (Fig. 4, C and D). Taken together, these results strongly suggest that ATM is required for 53BP1 hyperphosphorylation after γ-radiation.

53BP1 Is a Substrate of ATM In Vitro

S/TQ sites have been described to be the minimal essential recognition sites for ATM (Kim et al., 1999). 53BP1 contains a total of 30 S/TQ sites, many of them clustered in the

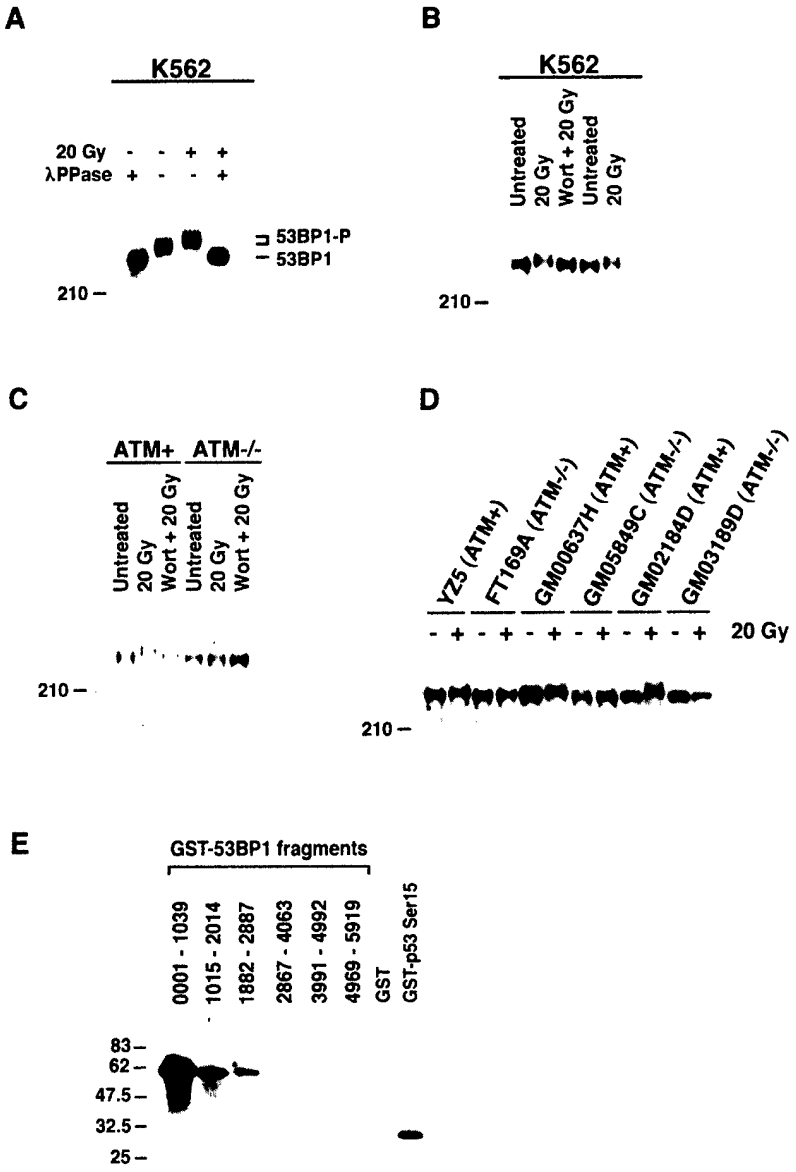

Figure 4. γ-Radiation–induced hyperphosphorylation of 53BP1 requires ATM. (A) γ-Radiation induces hyperphosphorylation of 53BP1. K562 cells were exposed to 0 or 20 Gy of γ-radiation and immunoprecipitated using polyclonal anti-53BP1 antibody. Immunoprecipitates were incubated for 1 h at 30°C with 800U λ-phosphatase (λPPase) in 100 μl incubation buffer or only incubation buffer. The samples were separated on a 3–8% gel and immunoblotted with anti-53BP1. (B) Wortmannin inhibits 53BP1 hyperphosphorylation. K562 cells were pretreated with 50 μM wortmannin for 30 min before exposure to 1 Gy of radiation. Whole cell lysates prepared from treated and control samples were separated on a 3–8% gradient gel (30 μg protein per lane) and immunoblotted with anti-53BP1 antibodies. (C) ATM is required for the γ-radiation–induced hyperphosphorylation of 53BP1. ATM-deficient GM03189D cells or ATM wild-type GM02184D cells were treated as described in the legend to B, and 30 μg lysates were separated on a 3–8% gel before immunoblotting with anti-53BP1 antibodies. (D) All three ATM-deficient cell lines tested (FT169A, GM03189D, and GM05849C) show no hyperphosphorylation 1 h after 20 Gy. (E) 53BP1 is a substrate of ATM in vitro. Six GST fusion proteins containing overlapping 53BP1 fragments were used as substrates in an ATM in vitro kinase assay. GST protein alone and GST fusion protein containing 13 residues surrounding the serine-15 of the p53 coding sequence were used, respectively, as negative and positive controls.

NH_2-terminal region. To examine whether 53BP1 is a substrate for ATM, and to define regions that can be phosphorylated by ATM in vitro, we designed six overlapping 53BP1 GST fragments that span the entire ORF of 53BP1 and performed a standard ATM kinase assay. As shown in Fig. 4 E, the first three NH_2-terminal 53BP1 fragments were phosphorylated by ATM in vitro, whereas no phosphorylation was observed in the last three COOH-terminal fragments, despite the fact that there are a total of 10 S/TQ sites within these 53BP1 fragments. These data suggest that 53BP1 is a substrate of ATM kinase.

Discussion

Here we report that 53BP1 participates in the early DNA damage–response. Using several antibodies specifically recognizing 53BP1, we show that 53BP1 becomes hyperphosphorylated and forms nuclear foci after exposure to ionizing radiation. γ-Radiation–induced 53BP1 hyperphosphorylation and foci formation are reduced in ATM-deficient cells. Moreover, 53BP1 hyperphosphorylation, as well as foci formation, is inhibited by wortmannin, an inhibitor of the PI3K-related kinases including ATM, DNA-PK, and, to a lesser extent, ATR (Sarkaria et al., 1998). Taken together, these data suggest that ATM and other PI3K-related kinases directly phosphorylate 53BP1 and regulate its localization to the sites of DNA strand breaks.

In favor of a functional link between ATM and 53BP1, we also demonstrate that NH_2-terminal fragments of 53BP1 are effectively phosphorylated by ATM in vitro. Similarly, Xia and colleagues have recently shown that *Xenopus* 53BP1 and a NH_2-terminal fragment of human 53BP1 can be phosphorylated by ATM in vitro and in vivo (Xia et al., 2000), supporting our hypothesis that 53BP1 is a direct substrate of ATM. In contrast to our findings, Schultz et al. (2000) observed no difference in 53BP1 foci formation in ATM-deficient cells when compared with that in normal ATM wild-type cells (Schultz et al., 2000). We also observed that 53BP1 foci still formed, albeit with slower kinetics, in cells lacking ATM, suggesting the exist-

ence of an alternative, ATM-independent pathway for the regulation of 53BP1. However, our data presented here clearly demonstrate that ATM plays a critical role in the regulation of 53BP1 hyperphosphorylation and foci formation after γ-radiation.

53BP1 rapidly colocalizes with γ-H2AX in response to ionizing radiation. H2AX is a histone H2A variant that becomes phosphorylated and forms foci at sites of DNA strand breaks after DNA damage (Rogakou et al., 1999; Paull et al., 2000). The number, as well as appearance and disappearance of 53BP1 foci, matched almost completely with that of γ-H2AX. Moreover, 53BP1 and γ-H2AX physically interact after ionizing radiation, suggesting that 53BP1 relocates to the sites of DNA double strand breaks in response to γ-radiation. Similar to 53BP1, γ-H2AX foci formation is inhibited by wortmannin treatment (Rogakou et al., 1999; Paull et al., 2000) and is reduced in ATM-deficient cells (Rappold, I., and J. Chen, unpublished observation). It is possible that phosphorylation of H2AX may mediate the relocalization of 53BP1 to DNA strand breaks. If this is the case, ATM-dependent hyperphosphorylation of 53BP1 may be a secondary event that is not required for 53BP1 foci formation. This possibility will be examined in future studies using phosphorylation-deficient mutants of 53BP1.

Upon relocalizing to the sites of DNA damage, 53BP1 could participate in chromosome remodeling that makes DNA lesions accessible to DNA repair proteins. Alternatively, 53BP1 could be involved in the recruitment of repair proteins like BRCA1 and Rad51 to these DNA lesions. Both of these proteins colocalize with 53BP1 several hours after exposure to ionizing radiation (Rappold, I., and J. Chen, unpublished observations). In addition, BRCA1 biochemically interacts with 53BP1 after γ-radiation (Rappold, I., and J. Chen, unpublished observations).

53BP1 contains two BRCT motifs at its COOH terminus. 53BP1 BRCT motifs are closely related with those of BRCA1 and *Saccharomyces cerevisiae* Rad9 (scRad9) protein. Insight into the potential role of scRad9 comes from studies of its association with scRad53. ScRad53 is the homologue of mammalian Chk2 or *Schizosaccharomyces pombe* Cds1. After DNA damage, scRad9 is phosphorylated and this phosphorylated scRad9 associates with the forkhead homology–associated (FHA) domain of scRad53 (Sun et al., 1998; Vialard et al., 1998). Mutations in either the scRad53 FHA domain (Sun et al., 1998) or scRad9 BRCT motifs (Soulier and Lowndes, 1999) prevent scRad53 activation after DNA damage. Although the mammalian homologue of scRad9 has not been identified, a scRad9 homologue likely exists in mammals. Because of the close homology of their BRCT motifs, two candidate scRad9 homologues are BRCA1 and 53BP1. Based on yeast studies, one would predict that the activation of Chk2, the homologue of scRad53, should depend on this scRad9 homologue in mammalian cells. However, DNA damage–induced phosphorylation of Chk2 was observed in BRCA1-deficient cells (Matsuoka et al., 1998), suggesting that BRCA1 may not be the mammalian homologue of scRad9. Experiments using 53BP1-deficient cells will be performed to examine whether 53BP1 is the scRad9 homologue in mammals.

In conclusion, our data demonstrate that 53BP1 participates early in DNA damage–signaling pathways and is regulated by ATM after γ-radiation. The exact role of 53BP1 in these pathways remains to be resolved. Given the importance of these DNA damage–signaling pathways in cancer prevention, it will be interesting to examine whether 53BP1 is mutated in tumors.

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